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(54) Title: ANTIBODY CONTAINING ELECTRODE

(57) Abstract

The invention pertains to a new process for producing polymeric electrodes incorporating macromolecular binding partners, and their use in electrochemical assays. Electrodes comprised of conducting polymers and macromolecules having the capacity to specifically bind particular molecules are also described. Electrodes produced in accordance with the invention may be used for determining the presence of particular molecules or for removing those molecules from solution. The invention has particular application to biosensor technology which may be used *in vivo* or *in vitro*.

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Title: "ANTIBODY CONTAINING ELECTRODE"

The present invention provides an electrosynthetic method for the direct incorporation of macromolecular binding partners into conducting polymeric coatings, for use in electrochemical assays and new electrodes containing one partner of a macromolecular binding pair.

Background

To date, most applications of electrochemical binding assays have centred on heterogeneous immunoassay systems employing enzyme labels, where the product of the enzyme-substrate interaction is monitored using high performance liquid chromatography with electrochemical detection (HPLC-ED) on flow injection analysis with electrochemical detection (FIA-ED) Henineman, W.R.; Halsall, H.B. Anal. Chem. 1985, 57, 1321A. In recent papers, the group of Smyth et al. have reported on the application of adsorptive stripping voltammetry (AdSV) to monitor directly in solution the reaction of human

serum albumin (HSA) with anti-HSA mouse immunoglobulin G (IgG) with anti-mouse IgG and concanavalin A with mannose Rodriguez Flores, J.; Smyth, M.R. J. Electroanal. Chem. 1987, 235, 317, Smyth, M.R.; Buckley, E.; Rodriguez Flores, J. Analyst, 1988, 113, 31, Rodriguez Flores, J.; O'Kennedy, R.; Smyth, M.R. Biosensors (submitted). In these studies, it has been demonstrated that the protein molecules are strongly adsorbed at the electrode surface, and that the magnitude of the electrochemical response is greatly influenced by the potential at which adsorptive accumulation is carried out.

Another area of increasing interest in electroanalysis is that of chemically modified electrodes, which offers the possibility of increased sensitivity and selectivity for the detection of certain analytes Wallace, G.G. in "Chemical Sensors". Blackie and Son. Glasgow, 1988. In particular, the area of polymer-modified electrodes has received much attention to date, and the group of Wallace et al. have concentrated their efforts on electrochemically synthesised polymers. It has already been demonstrated that the incorporation of various chemical reagents into such polymers is possible depending on the presence of charged sites and size and the incorporation of an enzyme (glucose oxidase) into polypyrrole has previously been reported Wallace, G.G.; Yuping, L. J. Electoanal.

Chem. (In Press). Umana, M.; Waller, J. Anal. Chem. 1986, 58, 2979; Wallace, G.G.; Imisides, M.D.; O'Riordan, D.M.T. Electrochem., Sensors and Analysis, Elsevier, Amsterdam, 1987, 293. Wallace, G.G.; O'Riordan, D.M.T. Anal. Chem . 1986, 58. Wallace, G.G.; Imisides, M.D. J. Electroanal. Chem. (In Press).

The aim of the present invention is to provide biosensor technology which might be used either in vivo or in vitro for the direct analysis of substances using bisospecific interaction, although other applications of the materials produced are possible.

Summary of the Invention

In one aspect, the invention provides electrodes comprising a conductive polymer having one partner of a macromolecular binding pair (binding partner) incorporated therein.

In a second aspect, the present invention provides a method of producing binding partner containing electrochemically generated polymeric film wherein polymer-binding partner electrodes are grown directly from solutions of monomer and binding partner molecules.

In a third aspect, the invention provides a method for the in vivo detection of the complement of the binding partner by use of a binding partner-containing electrode.

The binding partners preferred for use in the present invention are marcromolecules which bind

specifically with high affinity in a predominantly irreversible manner to particular complementary molecules. Either one of each of the partners of the followig pairs are particularly preferred:

antibody	-	antigen
receptor	-	hormone
lectin	-	carbohydrate
nucleic acid	-	nucleic acid (base pairs)

Preferably, the conductive polymeric electrode is an electrochemically generated polypyrrole, polyaniline, film, preferably generated at a platinum, gold or carbon electrode surface. However, polymerisation can be carried out on non-conductive substrates.

It may also be desirable that the polymerisation process be carried out in the presence of at least one surfactant to enhance the degree of binding partner incorporation and to coat the polymer backbone to minimise non-specific interations during the polymer bound binding partner-complementary binding partner reaction stage.

The use of surfactants also enables the use of monomers such as thiophene normally insoluble in aqueous solutions.

Similarly, other blocking proteins may be included in the polymerisation solution when producing the polymer to minimise non-specific interactions.

An alternative process is to prodcue a preliminary polymer coating with surfactants followed by coating the polymer containing binding partner over the preliminary coating. Such a sandwich technique may be useful in enhancing incorporation of the binding partner.

A further alternative process is to attach the binding partner to colloidal gold particles to enhance incorporation and orientation. This has been found to be particularly appropriate for use when the binding partner has no net negative charge, e.g. some types of antibody. The conductivity of the polymer may also be increased by incorporation of colloidal gold particles.

Colloidal gold particles may be synthesised in a range of sizes from 2nm to 1500nm. Such particles may be coated with a wide variety of substances, eg. antibodies, antigens, lectins, complex carbohydrates as well as smaller molecules such as amino acids, sugars etc. Such particles precoated with binding partners may be incorporated into polymers and can have beneficial effects in facilitating incorporation of molecules that are otherwise difficult to incorporate, such as those that carry a net positive charge at the pH preferred for other reasons of incorporation.

Additionally, colloidal gold particles need not be precoated with the substance but may be merely added at the time of electrosynthesis. Such addition causes the polymer to increase in conductivity during growth as

shown by a decrease in potential during galvanostatic growth.

Non-specific hydrophobic interations may be minimised by using monomers with hydrophilic groups.

In another aspect binding partners with a plurality of binding sites, such as antibodies, may be attached to the electrode surface by just incorporating the complement molecule into the polymer and then attaching the binding partner via the appropriate binding partner-complement interaction. This process is particularly appropriate for incorporating antibodies.

In another aspect of the invention, electrochemical responses can be enhanced by incorporating dispersed metal particles, such as mercury, throughout the polymer.

The synthesis of electrodes may be improved by the addition of 0.01 to 0.1M Tyron ® which catalyses growth at a lower potential and increases incorporation of the binding partner into the polymer.

The preferred synthesis conditions for each binding partner differs. Factors to be taken into consideration include the number and type of ionic species (eg. buffering anions), pH, temperature, and other physico-chemical factors. Conditions need to be determined individually for each pair of binding partners.

The invention is particularly applicable for use with antibodies and more particularly for use with

antibodies to human serum albumin (anti-HSA), for medical or veterinary diagnostic purposes and for antibodies to legionella for bioenvironmental sensors.

Brief Description of the Drawings

In the accompanying drawings,

Figure 1 represents chronopotentiograms for

a) polypyrrole - anti-HSA

b) polypyrrole - Cl electrodes,

Figure 2 represents a cyclic voltammogram of a typical polypyrrole - anti-HSA electrode in 0.1M $NaNO_3$, scan rate of 50mV/sec.

Figure 3 represents a cyclic voltammogram of a typical

a) polypyrrole - anti-HSA electrode, and

b) polypyrrole - Cl electrode in 0.1M $NaNO_3$ with a scan rate of 50mV/sec,

Figure 4 shows scanning electron micrographs for

a) polypyrrole - anti-HSA, and

b) polypyrrole - Cl electrodes,

Figure 5 represents negative ion FAB mass spectra for

a) polypyrrole - anti-HSA, and

b) polypyrrole - Cl,

Figure 6a is a cyclic voltammogram showing the effect of soaking on a polypyrrole - anti-HSA electrode in HSA solution (50ppm) for 5 minutes recorded in 0.1M $NaNO_3$ with a scan rage of 50mV/Sec,

Figure 6b shows the cyclic voltammogram obtained after soaking a polypyrrole - anti-HSA electrode in HSA with applied potential of -0.80V for 5

minutes, recorded in 0.1M $NaNO_3$ with a scan rate of 50mV/Sec.

Figure 7a represents AC voltammograms of a polypyrrole chloride electrode scanned in phosphate buffer at (i) $\phi = 0°$ and (ii) $\phi = 90°$

Figure 7b represents AC voltammograms of a polypyrrole - anti-HSA electrode scanned in phosphate buffer at (i) $\phi = 0°$ and (ii) $\phi = 90°$

Figure 8 gives the amino acid analysis of polymer samples after hydrolysis has been used to confirm the presence of protein in the polymer

Figure 9a depicts a Fourier Transfer Infra Red Spectrograph of a conducting polymer film containing anti-HSA

Figure 9b depicts a Fourier Transfer Infra Red Spectrograph of the conducting polymer of Figure 9a containing chloride as the counterion to replace anti-HSA

Figure 10 shows the change in electrochemical properties of a polymer containing anti-HSA in the presence of HSA

Figure 11 shows the change in electrochemical properties of a polymer containing anti-legionella in the presence of legionella

Detailed Description of the Preferred Embodiments

Although the following examples relate only to polypyrrole and antibodies, it should be readily

understood by those skilled in the art that other conducting polymers and other binding partners may be substituted for those described and remain within the scope of the present invention.

Example 1

Polypyrrole- anti-HSA electrodes may be grown from solutions of pyrrole (0.1M - 0.5M) and anti-HSA (10 - 500mgl^{-1}).

Electrochemical polymerisation may be carried out at a platinum wire electrode, preferably using 0.5M pyrrole which is 10 - 500ppm in anti-HSA. Films produced in this manner are comparable in appearance to a polypyrrole chloride film grown in the presence of 0.1 - 1M Cl^-, thus indicating that the antibody is acting as a counterion, and that its presence is required for growth of the polymer at the electrode surface.

When a polypyrrole-anti-HSA (PP-anti-HSA) electrode was prepared (as above) with an antibody concentration of 100 ppm, it gave rise to the chronopotentiogram shown in Fig. 1a. The chronopotentiogram for a typical PP-Cl electrode grown under similar conditions is shown in Fig. 1b. From this it can be seen that the potential required to initiate polymerisation was greater for the PP-anti-HSA electrode than for the PP-Cl electrode. In both cases, the potential becomes constant with respect to time indicating continuing growth of a conducting polymer at the electrode surface. This was different

from the behaviour observed when a molecule such as EDTA was incorporated as the counterion, where the potential increased, indicating that a higher potential was required for further growth of a less conducting polymer Wallace, G.G.; Yuping, L. J.Electroanal. Chem. (In Press).

Solution conditions providing optimum incorporation and orientation such that antibodies remain active may be provided by using either phosphate buffer at pH 7.2 or water. Antibody concentrations up to 25 g/L may be used and are preferred in the polymerisation solution. Electrochemical conditions may also be optimised to ensure maximum interaction by using galvanostatic methods at low current densities (< 250$\mu A/cm^2$) or potentiodynamic methods scanning between 0.0V and =1.20V vs Ag.AgCl at 100 mv/sec for 1 to 3 scans. Short polymerisation times are also preferred as these produce thin polymers on which interactions are easier to monitor i.e. 12 minutes galvanostat growth or 1 scan potentiodynamically.

Cylic Voltammetry

When PP-anti-HSA electrodes (100 ppm in anti-HSA) were studied using cyclic voltammetry (CV) in 0.1M $NaNO_3$ solution over a potential range of -1.0V to +0.70 V, a peak (peak A) was observed at around -0.1V on the forward scan which disappeared on subsequent scans (Fig. 2). The loss of this response

may be attributed to denaturation of the protein at high positive potentials, as was demonstrated by Rogriguez Flores and Smyth in their paper dealing with the CV behaviour of HSA and anti-HSA at Hg electrodes. Rodriguez Flores, J.; Smyth, M.R. J. Electroanal. Chem. 1987, 235, 317.

The potential on the anodic side was therefore limited to +0.25V. A typical cyclic voltammogram of a PP-anti-HSA electrode (100 ppm in anti-HSA) operated using a potential range of -0.80 to +0.25 V is shown in Fig. 3a. From this it can be seen that peak A (at -0.07V) slowly shifts to more cathodic potentials and decreases in current on repetitive scanning, but retains most of its activity. The cyclic voltammetric behaviour of a typical PP-Cl electrode in the same supporting electrolyte is shown in Fig. 3b. From this it can be seen that the main peak seen on scanning for -0.80 to +0.25 V occurs at -0.20 V on the first scan, but shifts to more anodic potentials and decreases slightly in size on repetitive scanning. This peak can be attributed to an influx of NO_3^- ion into this polymer, as the polymer undergoes a transition from a less conducting to a more conducting state Wallace, G.G.; Imisides, M.D.; O'Riordan, D.M.T. Electrochem., Sensors and Analysis, Elsevier, amsterdam, 1987, 293. It is likely, therefore, that peak A seen with the PP-anti-HSA electrodes is also associated with this mechanism, and

that the differences in potential are due to structural differences in the two polymer films and/or the differences in mobility of the counterions (Cl^- and Anti-HSA). It can also be seen that the PP-Cl electrode is conductive over a greater potential range.

Comparison of AC voltammetry also indicated that there are significant differences between the PP/Cl and the PP/anti-HSA electrodes (Figs 7a and 7b).

Scanning Electron Microscopy

The scanning electron micrographs (SEM) obtained for the PP-anti-HSA and PP-Cl electrodes are shown in Fig. 4a and b respectively. A distinct difference in morphology is observed for polymers grown in the presence of the antibody.

FAB Mass Spectrometry

Negative ion spectra displayed higher molecular weight fragments not previously observed on polypyrrole materials.

Amino Acid Analysis

Amino Acid analysis of polymer samples after hydrolysis has been used to confirm the presence of protein in the polymer as shown in Fig. 8. Protein concentrations in excess of 2% w/w can be achieved.

Fourier Transfer Infra Red Spectroscopy

Fourier Transfer Infra-Red Spectroscopy has been used to monitor polymer films with and without protein present. The results are shown in Figs. 9a and 9b respectively.

Application of PP-anti-HSA electrode as a Sensor for Determination of HSA

When a PP-Cl electrode was placed in solutions of 10-100 ppm HSA in 0.1M $NaNO_3$ no significant change in CV behaviour was observed. When a PP-anti-HSA electrode was placed in solutions of varying HSA concentrations and then scanned in 0.1M $NaNO_3$, peak A was again seen on the forward scan at around -0.60V. This response decreased in size with repetitive scanning (Fig. 6a). When the PP-anti-HSA electrode was held at -0.80V for 5 min in a solution of 100 ppm HSA in 0.1M $NaNO_3$, peak B increased in size (Fig. 6b).

It would therefore appear that there is a specific interaction between the anti-HSA immobilised in the polymer and HSA which has diffused to the surface or through the polymer matrix. The interaction was found to be both time and potential dependent. The nature of this peak has not yet been ascertained. It may be due to reduction of the HSA, or may be the effect that the interaction of HSA with anti-HSA has on the polymer structure.

It has also been found that the use of AC voltammetry is appropriate in the electrochemical monitoring of binding partner-complement interactions as shown in Fig 10. These should be compared with the AC response in Fig 7b before interactions. Figure 10 is AC voltammograms in phosphate buffer after

interacting a polypyrrole - anti-HSA electrode with 10ppm HSA in solution for 30 minutes at (i) $\phi = 0°$ and (ii) $\phi = 90°$.

Figure 11 is equivalent AC voltammograms for legionella interaction as described in the following example.

Example 2

Legionella antibodies may be incorporated into polypyrrole polymer electrodes using potentiodynamic growth (OV to 1.2V at 100mV/sec for 1 to 3 scans) in a solution of 0.1M pyrrole with 50 ppm anti-legionella in a trisglycine buffer at a pH of 6.0.

The polymer may be deposited on a range of substrates, the preferred one being platinum.

After preparation the polymer containing the antibodies interacted with legionella at low concentrations in the order of 1,000 organisms/mL to alter the electrical properties of the polymer as shown in Fig. 11.

Although the above examples refer only to specific embodiments of the invention, it should be understood that the invention is applicable to other polymers and other binding partners.

Other suitable polymers which may be employed include polythiophene, polyaniline and polyfuran. Copolymers comprising the monomeric units of these polymers may also be used.

Claims

1. A polymeric electrode comprising a conductive polymer having at least one partner of a macromolecular binding pair incorporated therein.

2. A polymeric electrode according to claim 1 wherein the polymer is an electrochemically generated polymer generated at a platinum, gold or carbon electrode surface.

3. A polymeric electrode according to claim 2 wherein the polymer is selected from the group consisting of polypyrrole, polythiophene, polyaniline and polyfuran, derivatives thereof or copolymers comprising the monomeric units of the said polymers.

4. A polymeric electrode according to any one of claims 1 - 3 wherein the partner of a macromolecular binding pair is selected from the group consisting of antibodies, antigens, hormones, hormone receptors, lectins, carbohydrates, and nucleic acids having complementary sequences.

5. A polymeric electrode according to claim 4 wherein the polymer is polypyrrole and the partner of a macromolecular binding pair is an antibody.

6. A process for producing a polymeric electrode having incorporated therein one partner of a macromolecular binding pair comprising electrochemically generating polymeric film from solutions of monomer and binding partner molecules on a suitable substrate.

7. A process according to claim 6 wherein the electrochemical generation is carried out in the presence of a surfactant.

8. A process according to claim 6 wherein the said partner of a macromolecular binding pair is attached to colloidal gold particles prior to incorporation into said polymer.

9. A process according to claim 6 wherein the solution of monomer and binding partner molecules additionally contains colloidal gold particles.

10. A process according to claim 6 wherein the electrochemical generation is carried out in the presence of 0.01 - 0.1M Tyron.

11. A process for producing antibody containing polymeric electrodes comprising electrochemically generating a polypyrrole film at a platinum wire electrode from a solution of approximately neutral pH containing pyrrole and antibodies.

12. A process according to claim 11 wherein the antibodies are antibodies to Human Serum Albumin.

13. A process according to claim 11 wherein the antibodies are antibodies to Legionella.

14. A method of detecting the presence of the complement of a partner of a macromolecular binding pair comprising measuring the change in electrochemical properties of a polymeric electrode incorporating said partner in a solution suspected of containing said complement.

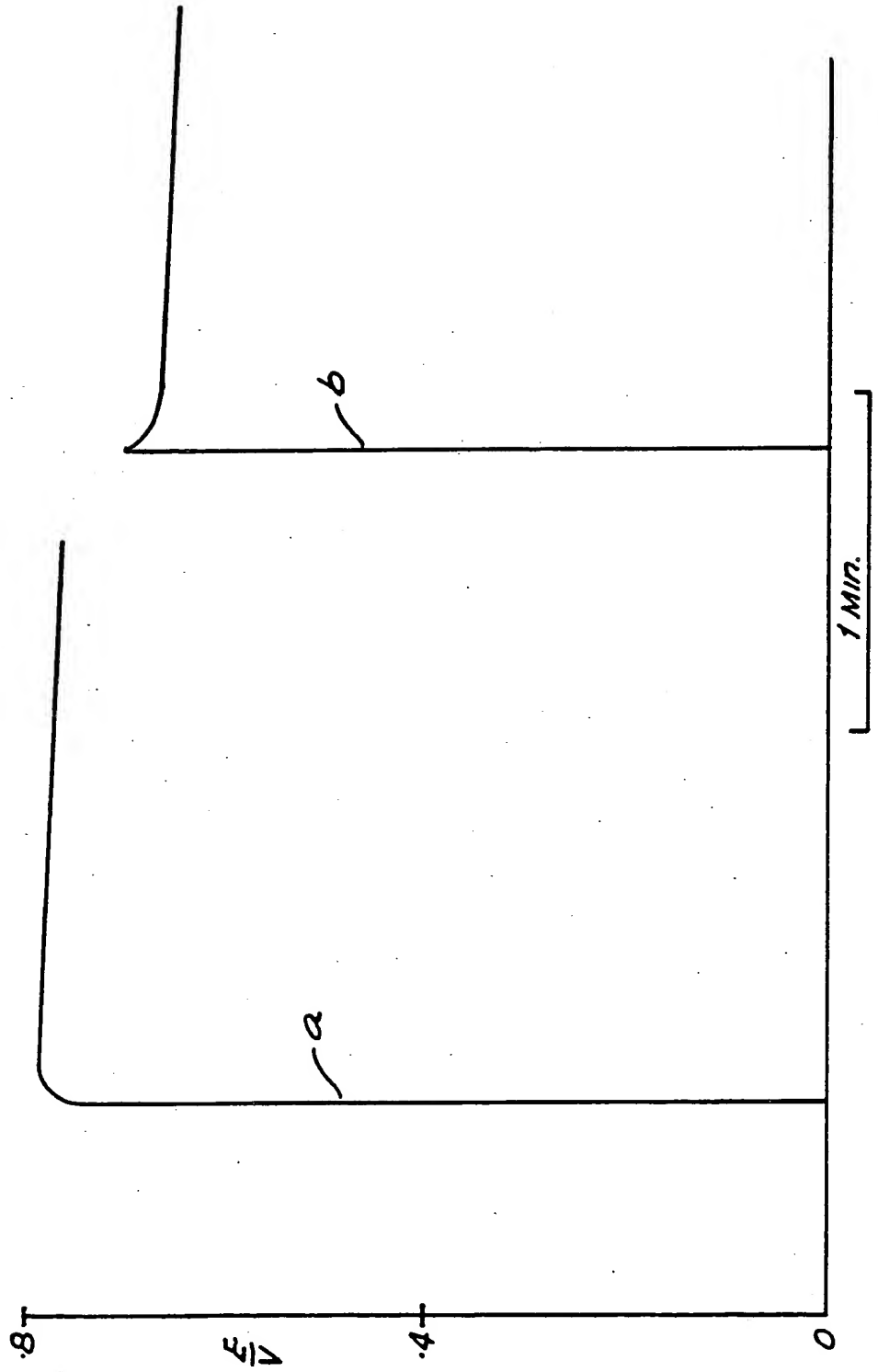

FIG. 1

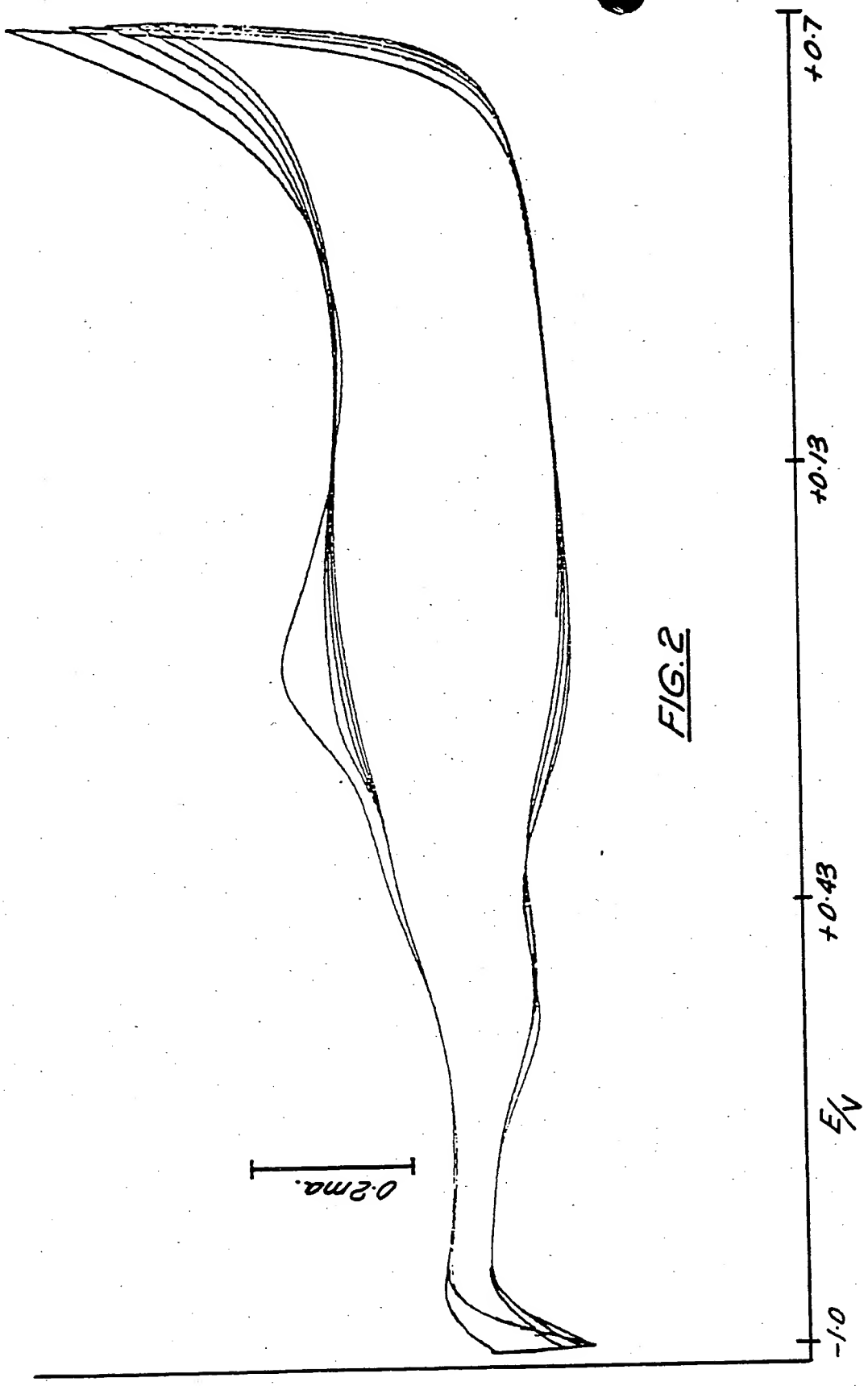

FIG.2

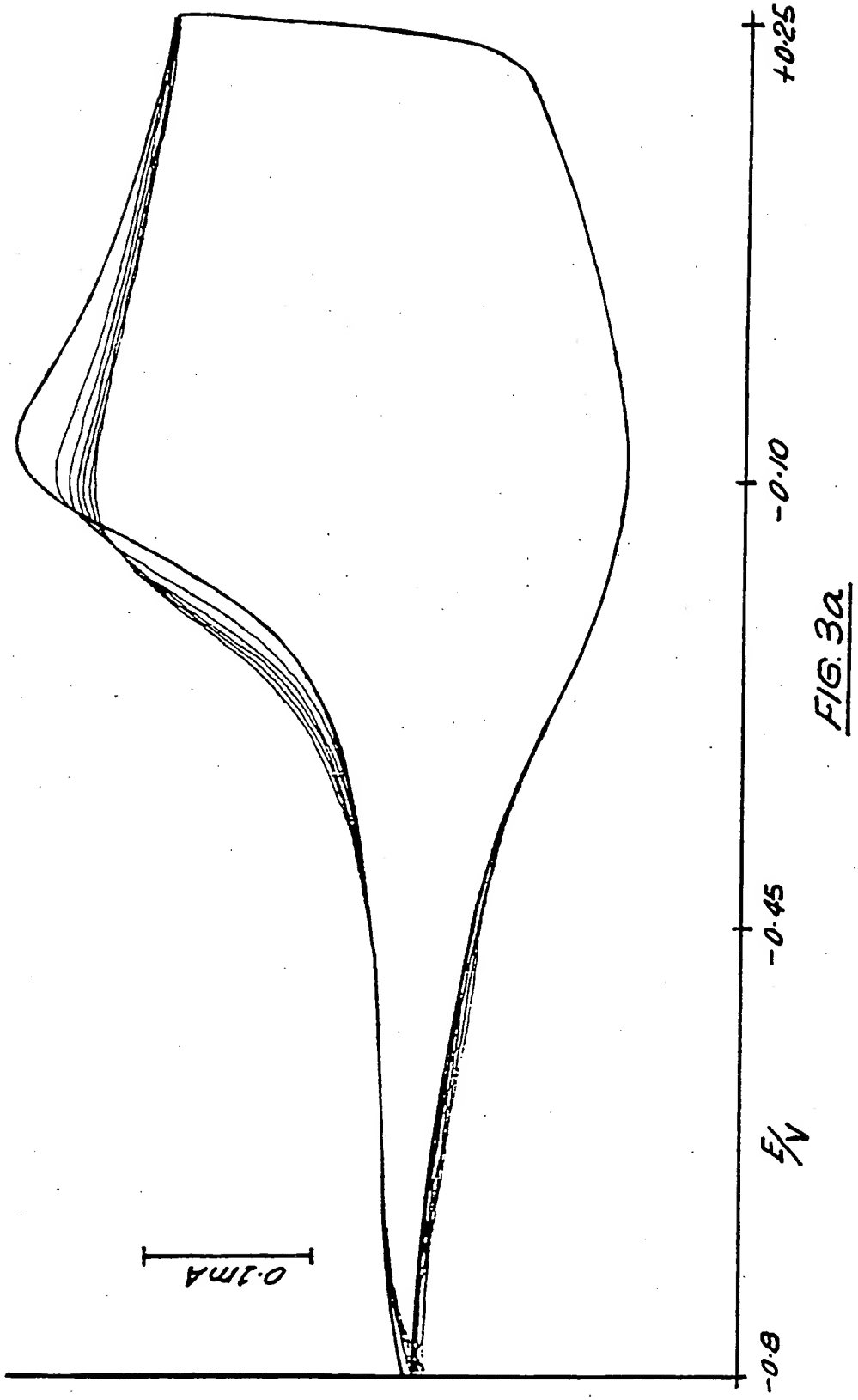

FIG. 3a

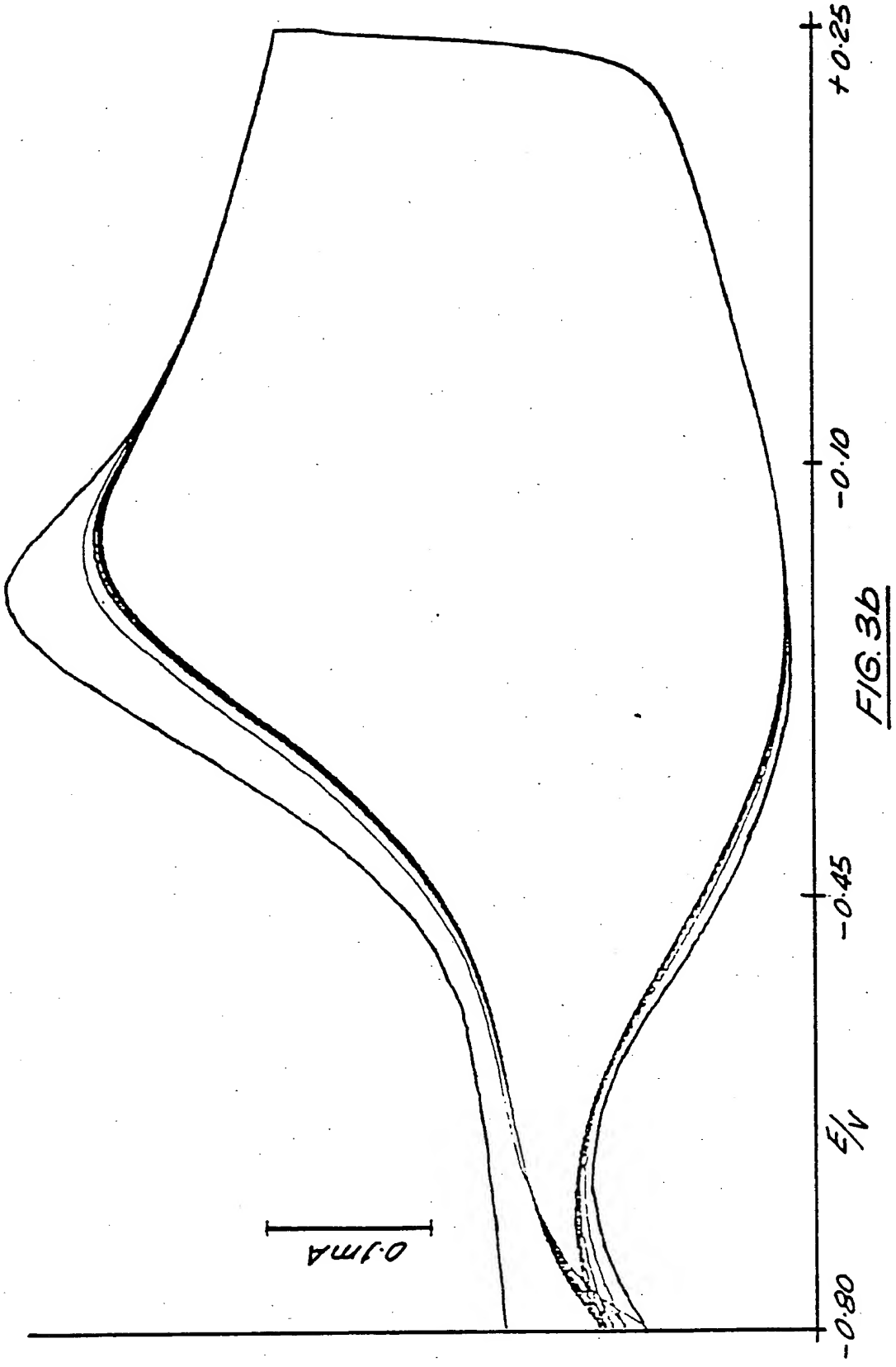

FIG. 3b

FIG. 4a

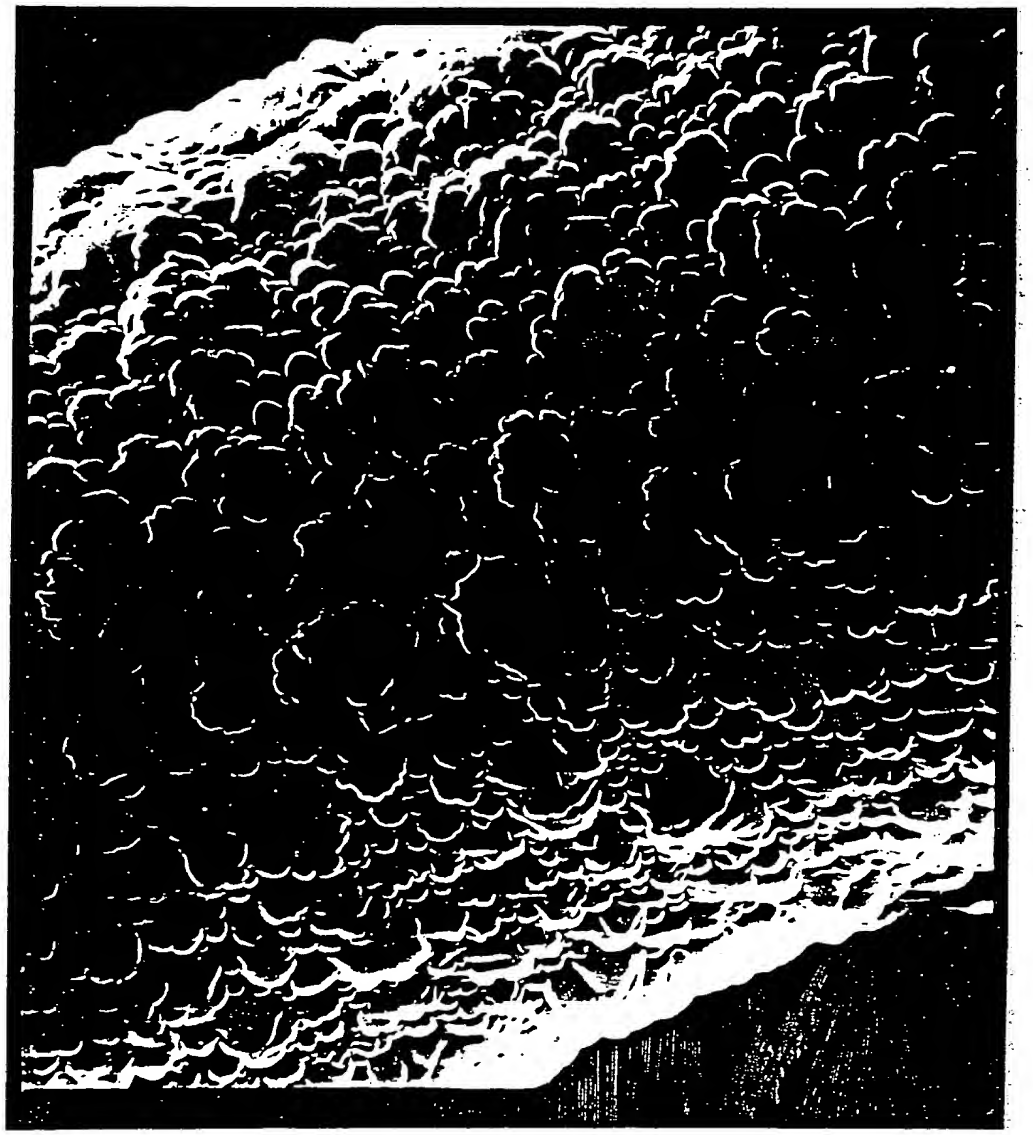

FIG. 4b

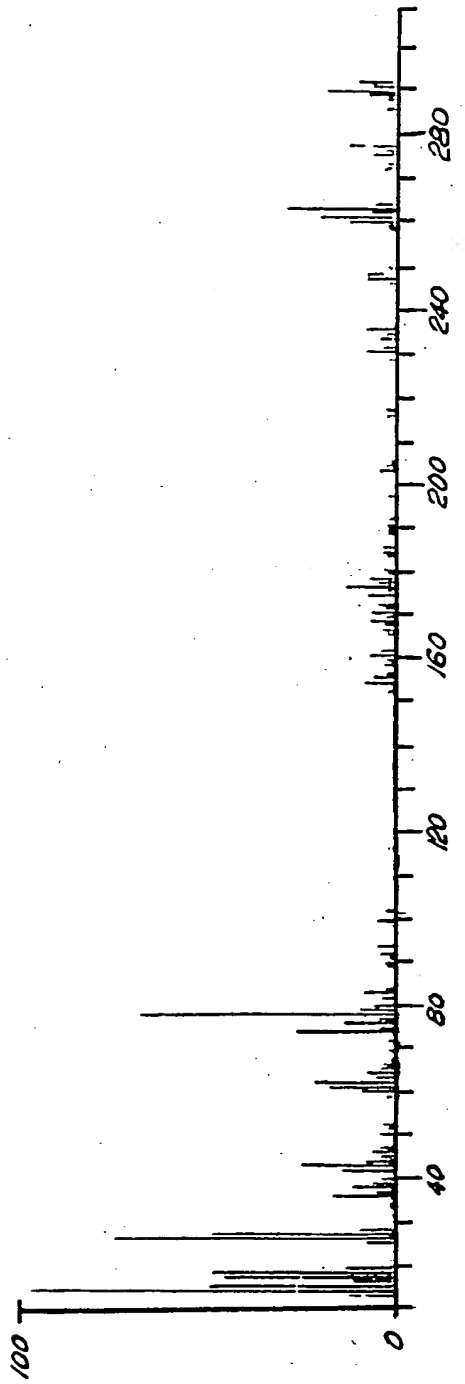

FIG. 5a

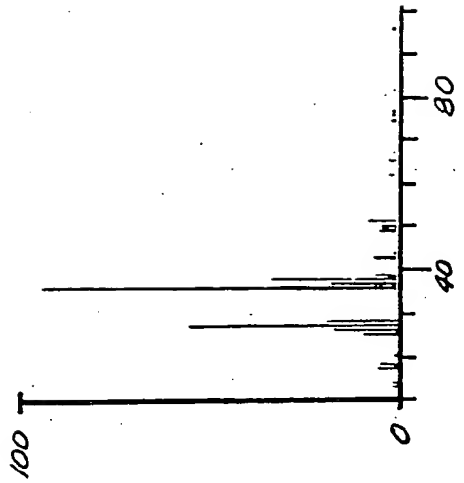

FIG. 5b

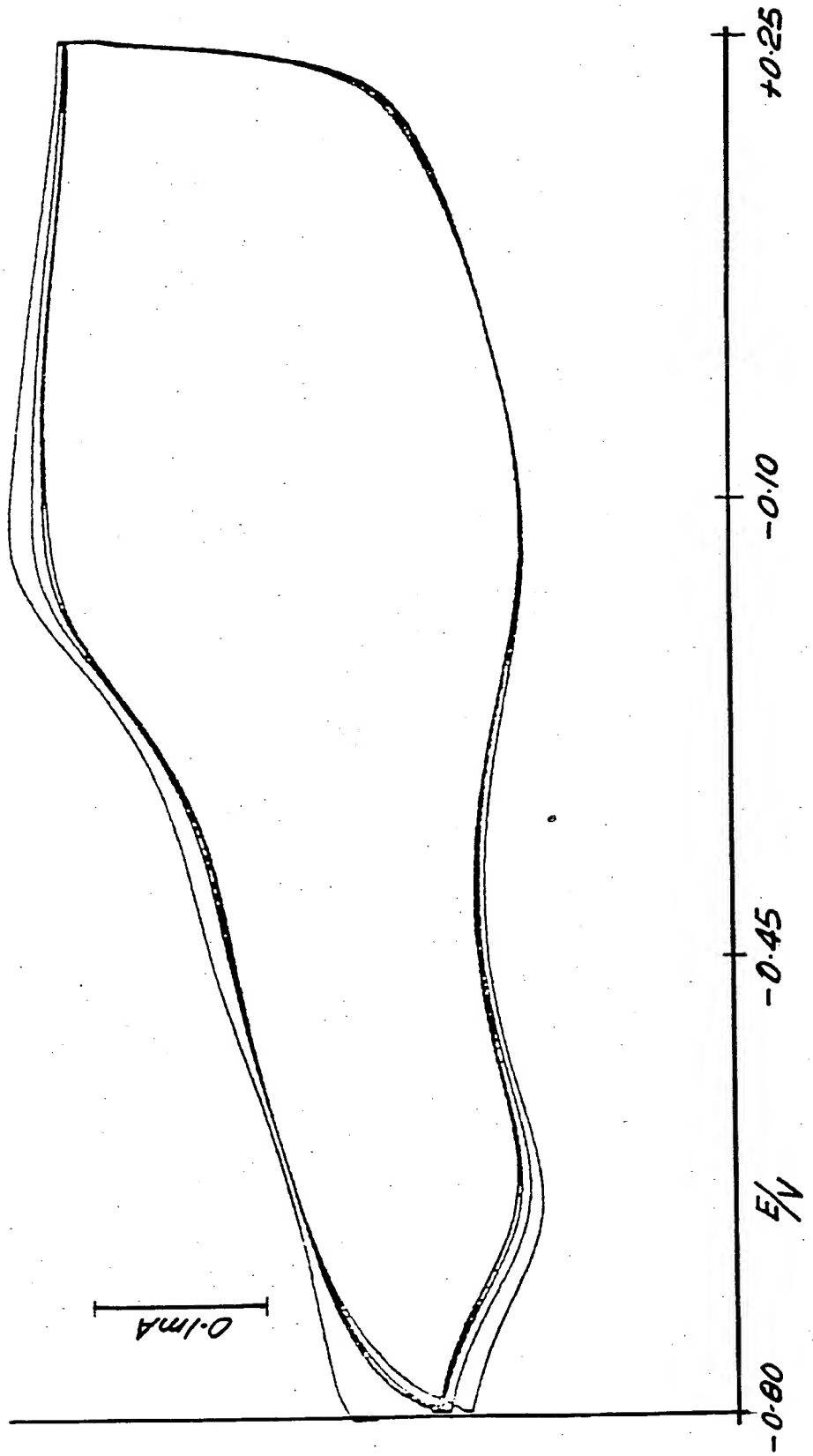

FIG. 6a

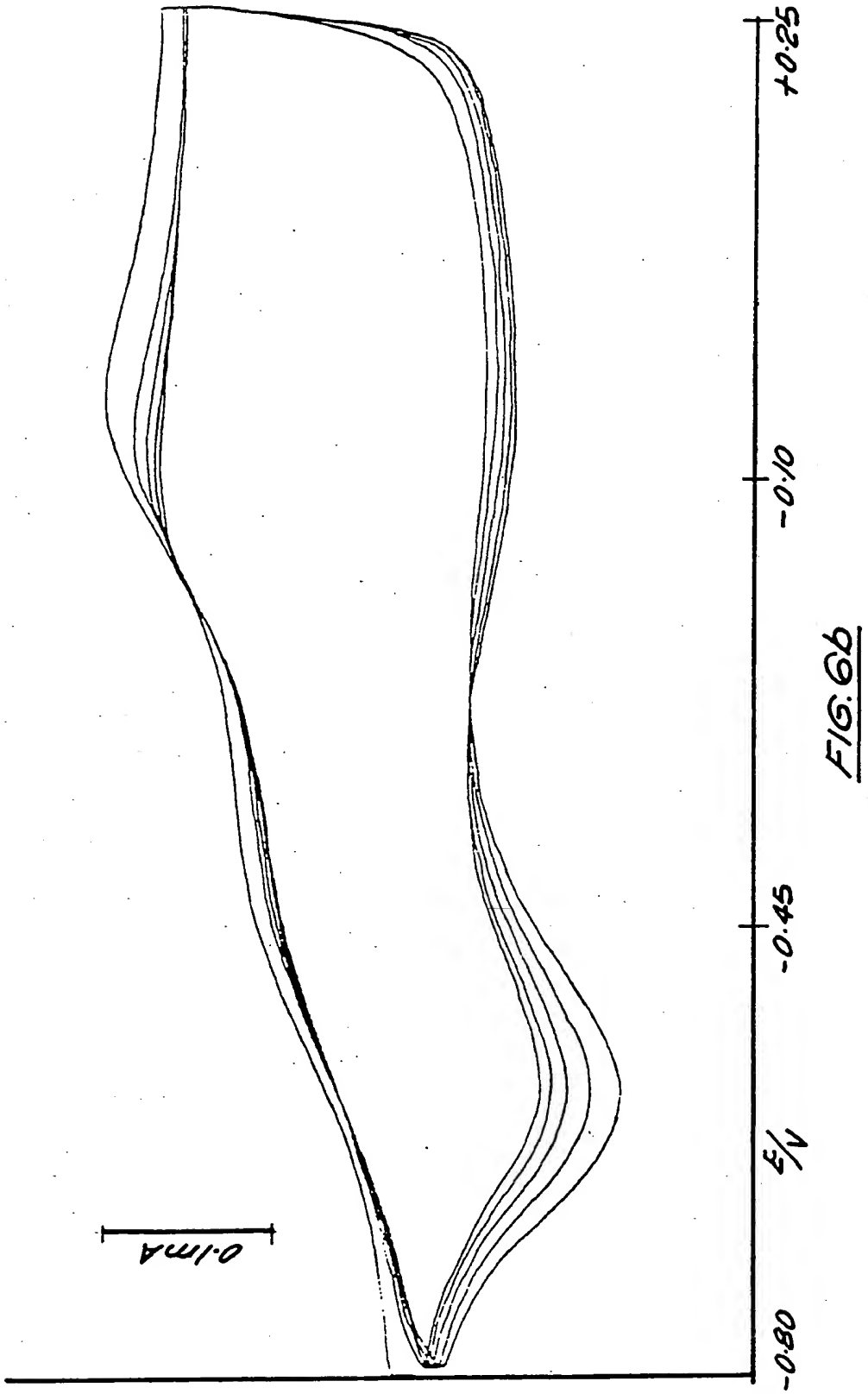

FIG. 6b

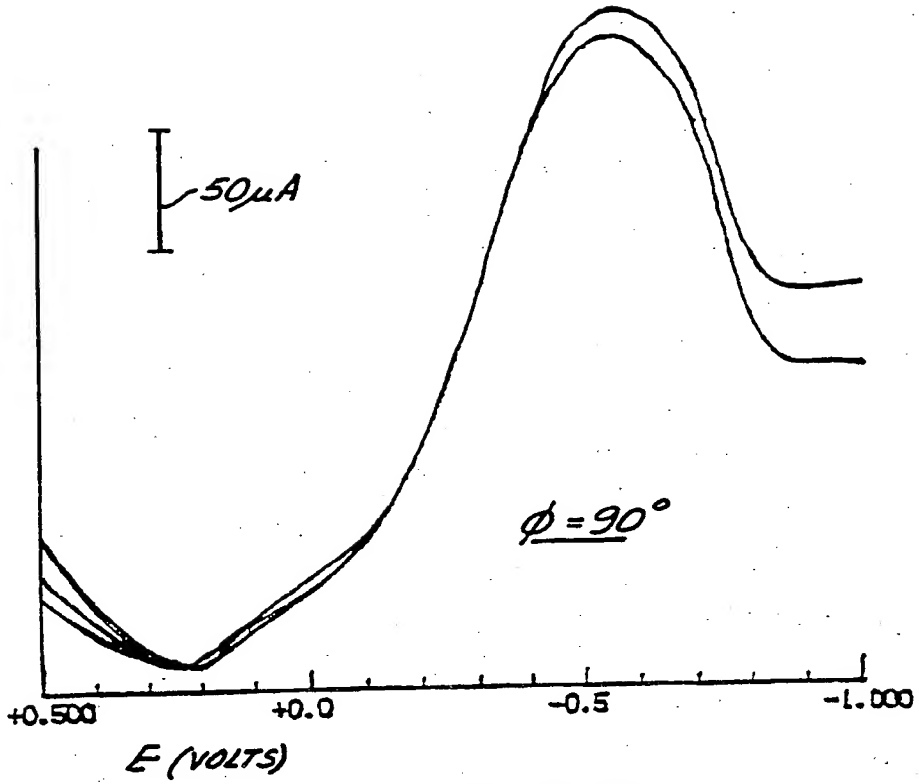

FIG. 7a(ii)

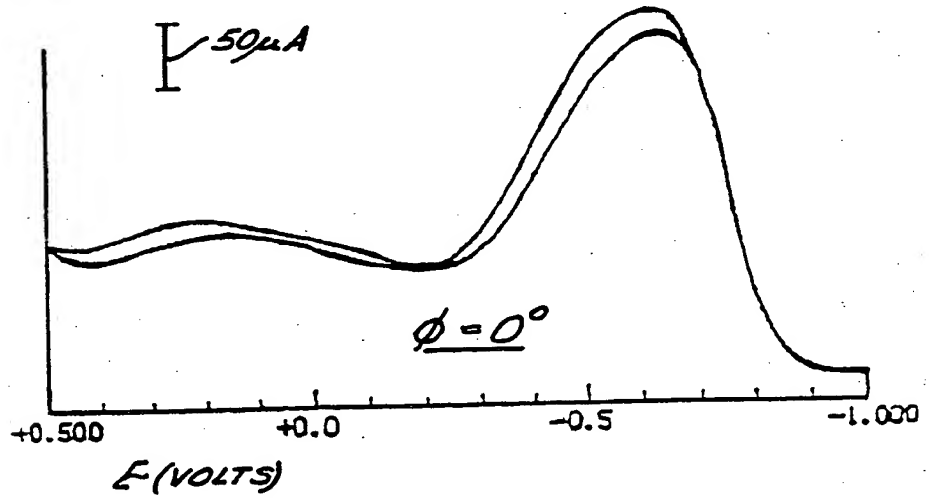

FIG. 7a(i)

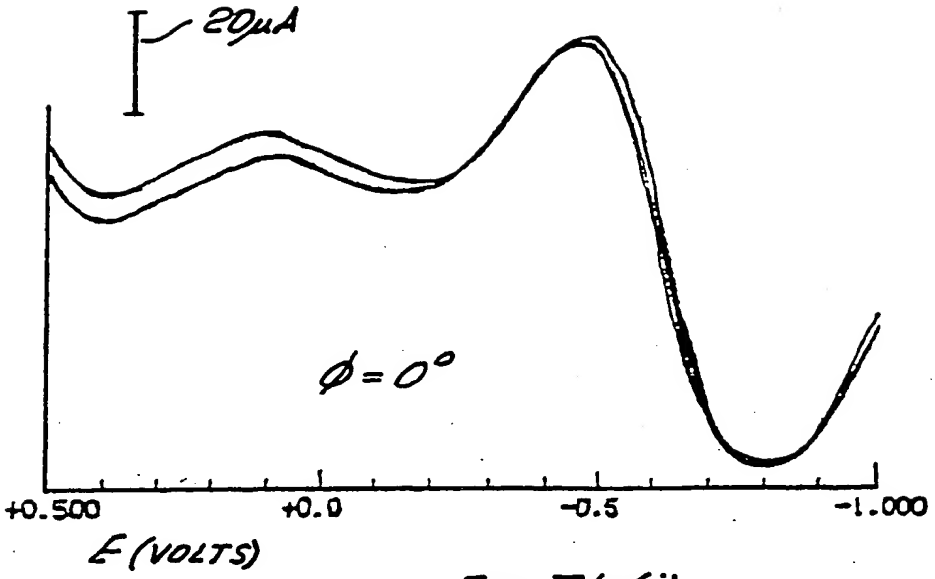

FIG. 7b(i)

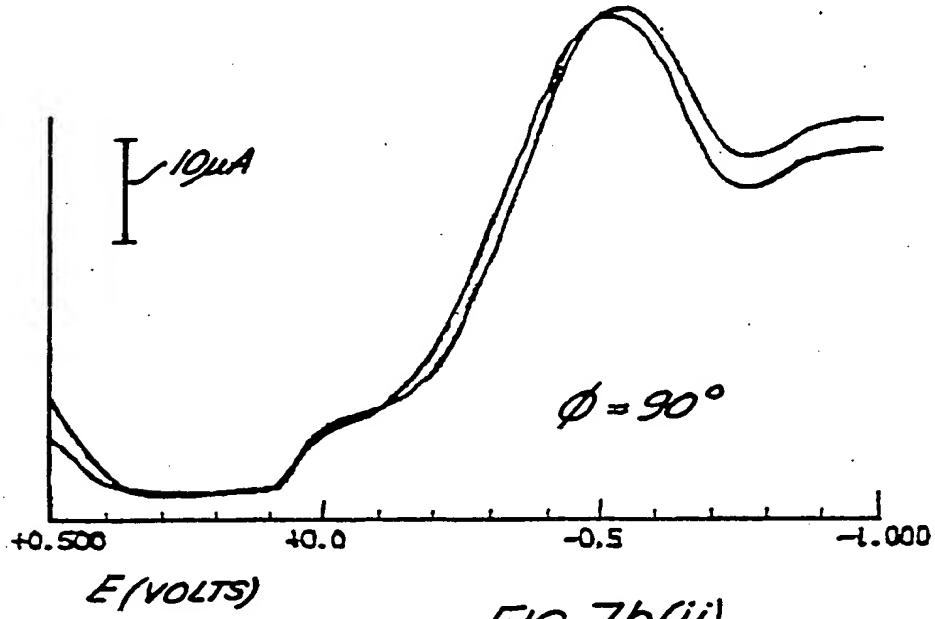

FIG. 7b(ii)

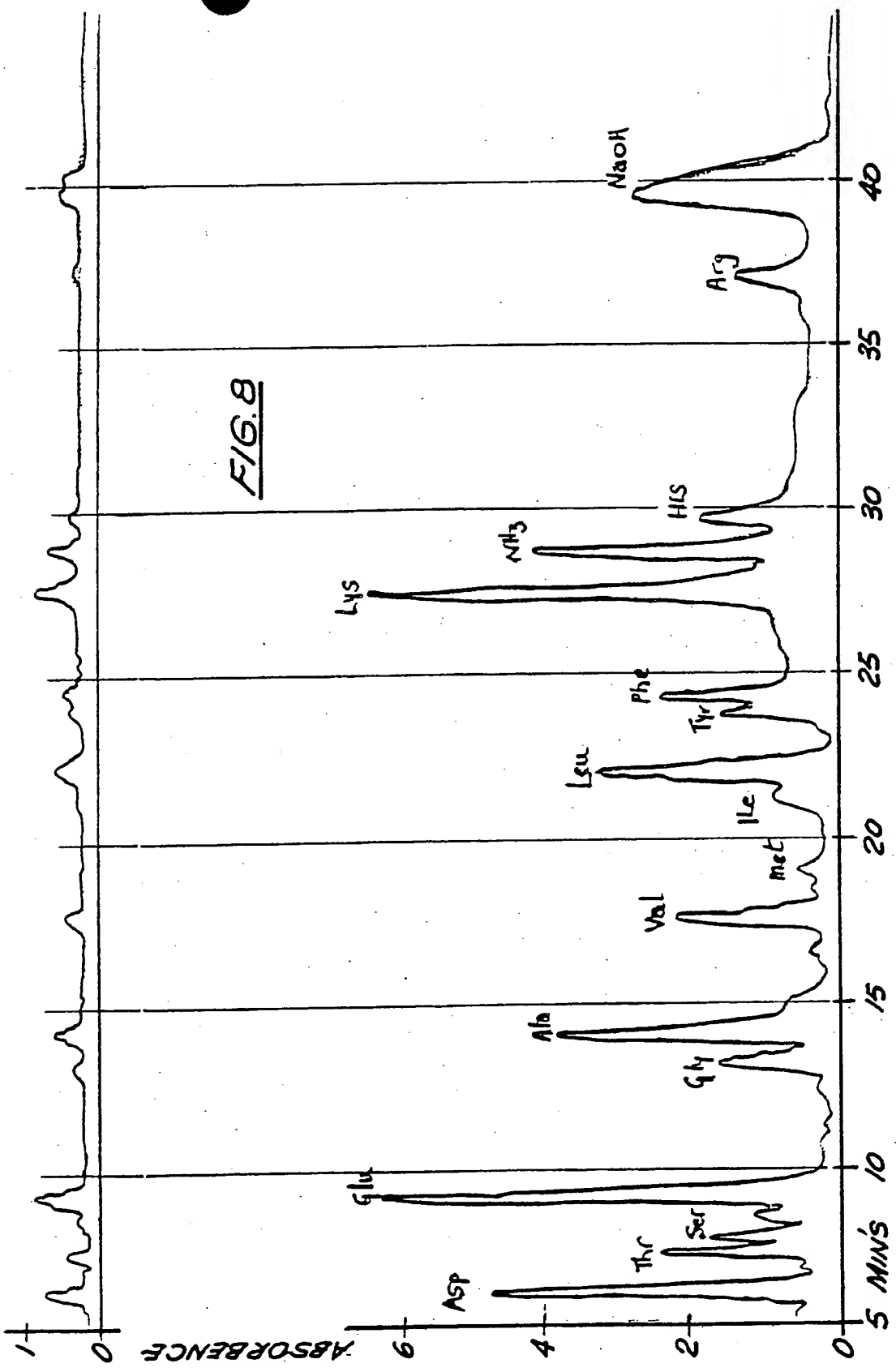
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FIG.8

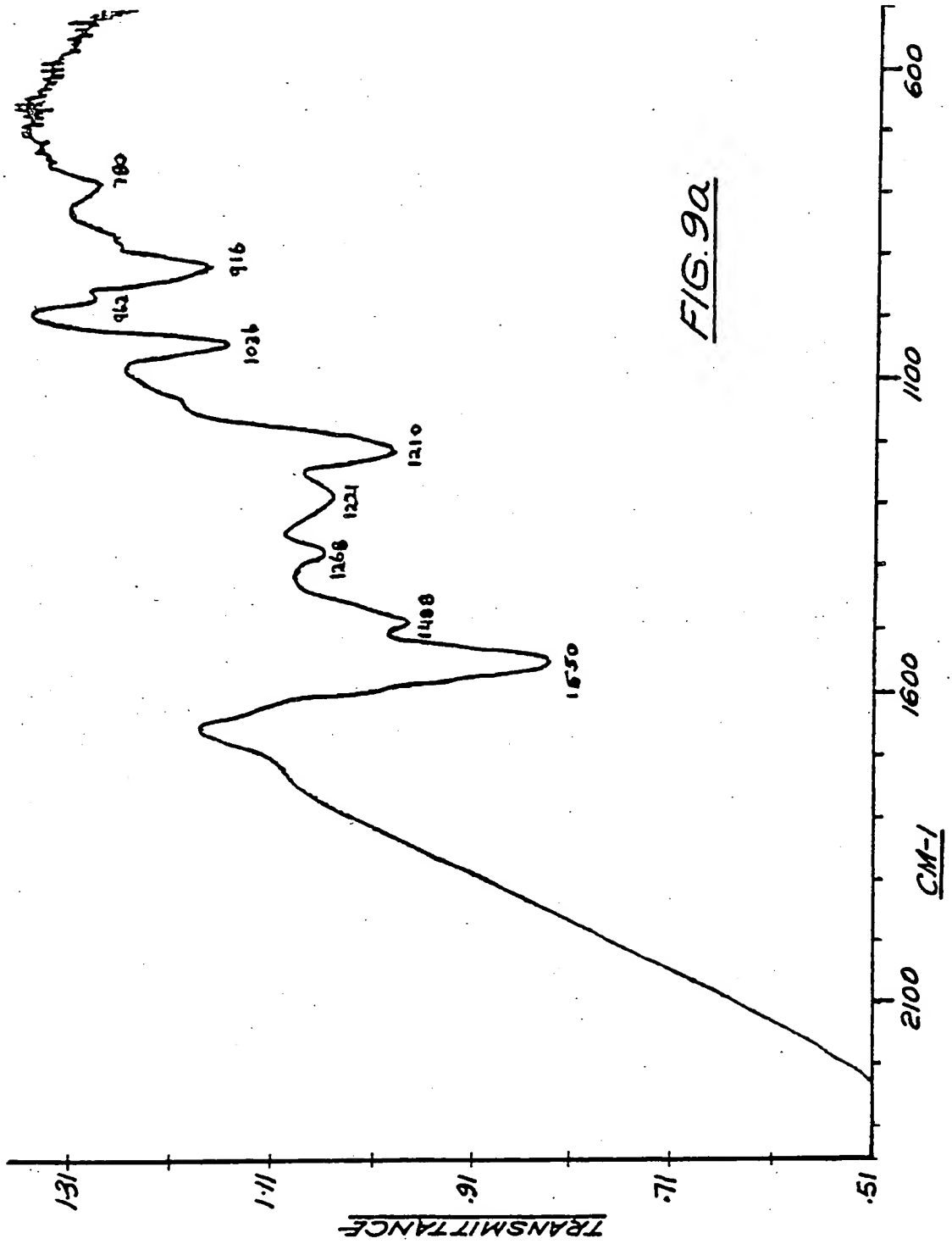

FIG. 9a

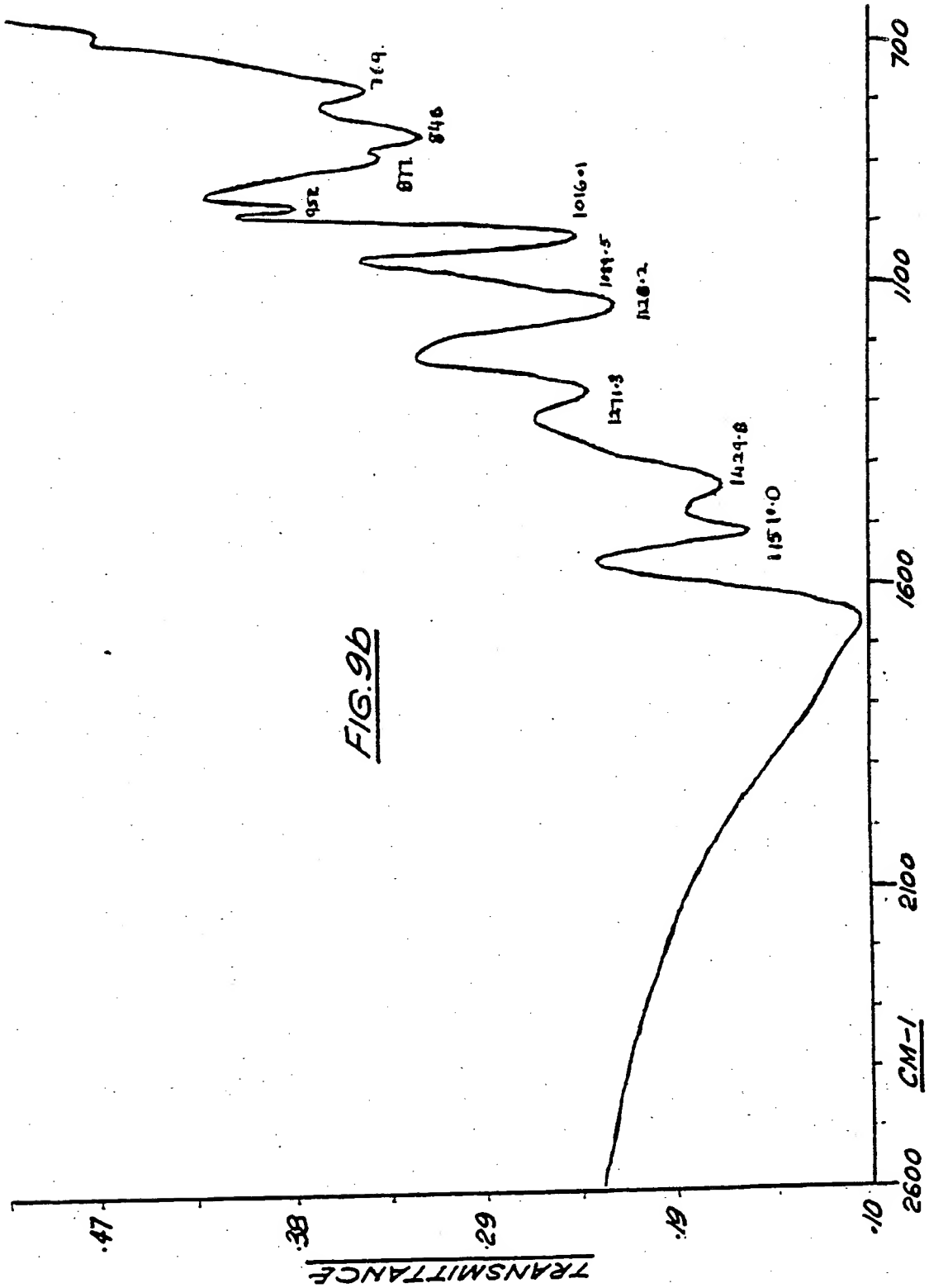

FIG. 9b

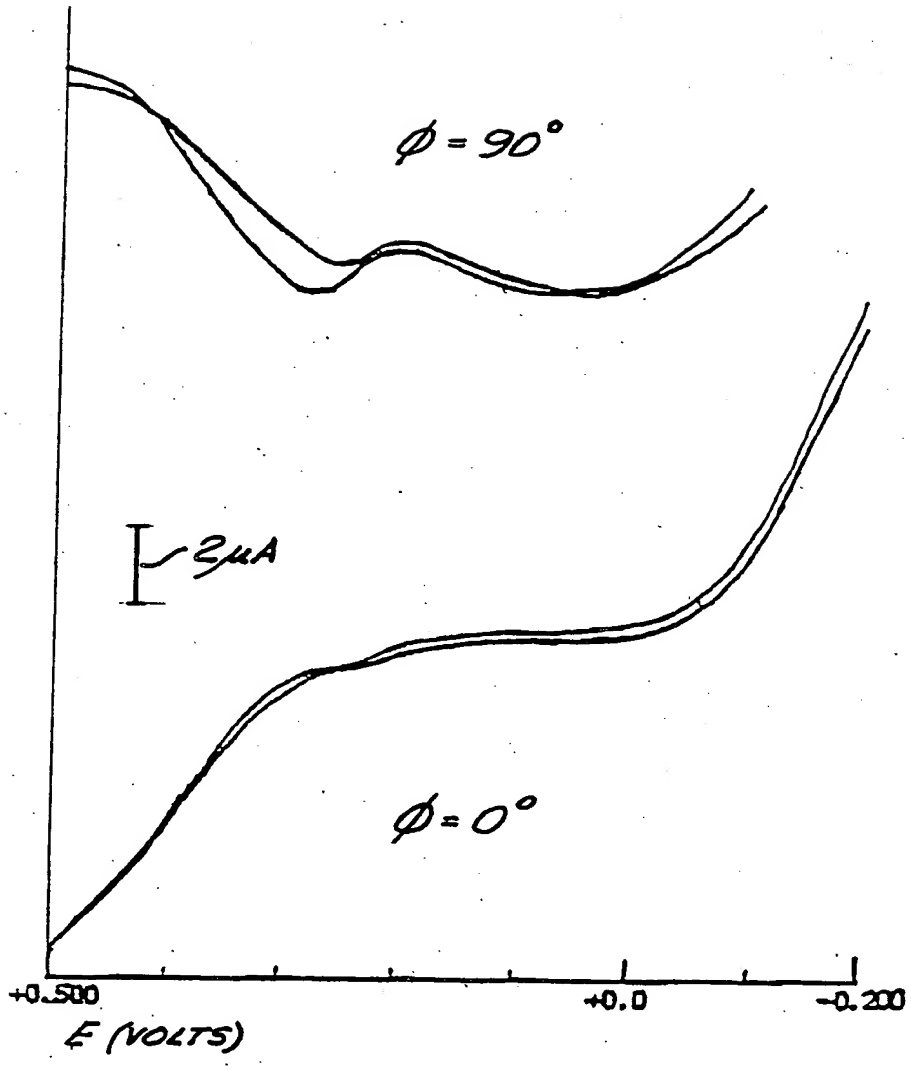

FIG. 10

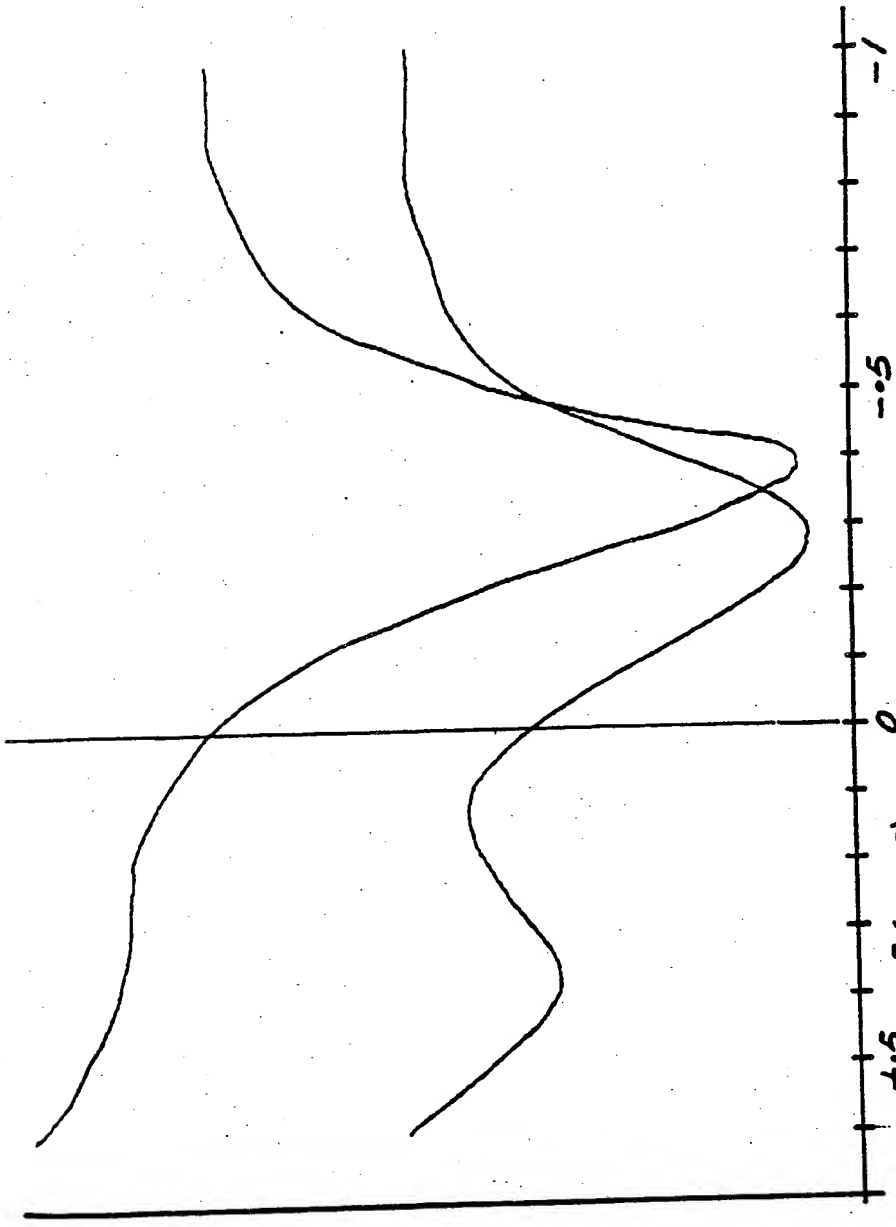

FIG. 11

I. TERNATIONAL SEARCH REP. T

International Application No. PCT/AU 89/00214

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] G01N 27/30

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	G01N 27/30

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	EP,A,193154 (JURIDICIAL FOUNDATION THE CHEMO-SERO-THERAPEUTIC RESEARCH INSTITUTE) 3 September 1986 (03.09.86) See Claims 1-9	1-14
X	Anal. Chem, Vol 58, published January, 1986 (Washington, USA), Umana & Waller "Protein-modified Electrodes", pp2979-2983	1-3
A	Anal Chem, Vol 58, published January, 1986 (Washington, USA), O'Riordan & Wallace "Poly (pyrrole-N-carbodithioate) Electrode for Electroanalysis" pp128-131	1-3

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31 August 1989 (31.08.89)	8 August 1989
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	ROBYN KNOCK Robyn Knock

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 89/00214

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members
EP 193154	CA 1241057 JP 61195346

END OF ANNEX